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# Effects of 25-Hydroxyvitamin D<sub>3</sub> on Proliferation and Osteoblast Differentiation of Human Marrow Stromal Cells Require CYP27B1/1 $\alpha$ -Hydroxylase

Shuo Geng,<sup>1,2</sup> Shuanhu Zhou,<sup>1</sup> and Julie Glowacki<sup>1</sup>

<sup>1</sup>Department of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

<sup>2</sup>Department of Orthopedic Surgery, First Affiliated Hospital of Harbin Medical University, Harbin Medical University, Harbin, Heilongjiang, People's Republic of China

## ABSTRACT

1,25-Dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] has many noncalcemic actions that rest on inhibition of proliferation and promotion of differentiation in malignant and normal cell types. 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulates osteoblast differentiation of human marrow stromal cells (hMSCs), but little is known about the effects of 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] on these cells. Recent evidence shows that hMSCs participate in vitamin D metabolism and can activate 25(OH)D<sub>3</sub> by CYP27B1/1 $\alpha$ -hydroxylase. These studies test the hypothesis that antiproliferative and prodifferentiation effects of 25(OH)D<sub>3</sub> in hMSCs depend on CYP27B1. We studied hMSCs that constitutively express high (hMSCs<sup>hi-1 $\alpha$</sup> ) or low (hMSCs<sup>lo-1 $\alpha$</sup> ) levels of CYP27B1 with equivalent expression of CYP24A1 and vitamin D receptor. In hMSCs<sup>hi-1 $\alpha$</sup> , 25(OH)D<sub>3</sub> reduced proliferation, downregulated proliferating cell nuclear antigen (PCNA), upregulated p21<sup>Waf1/Cip1</sup>, and decreased cyclin D1. Unlike 1,25(OH)<sub>2</sub>D<sub>3</sub>, the antiapoptotic effects of 25(OH)D<sub>3</sub> on Bax and Bcl-2 were blocked by the P450 inhibitor ketoconazole. The antiproliferative effects of 25(OH)D<sub>3</sub> in hMSCs<sup>hi-1 $\alpha$</sup>  and of 1,25(OH)<sub>2</sub>D<sub>3</sub> in both samples of hMSCs were explained by cell cycle arrest, not by increased apoptosis. Stimulation of osteoblast differentiation in hMSCs<sup>hi-1 $\alpha$</sup>  by 25(OH)D<sub>3</sub> was prevented by ketoconazole and upon transfection with CYP27B1 siRNA. These data indicate that CYP27B1 is required for 25(OH)D<sub>3</sub>'s action in hMSCs. Three lines of evidence indicate that CYP27B1 is required for the antiproliferative and prodifferentiation effects of 25(OH)D<sub>3</sub> on hMSCs: Those effects were not seen (1) in hMSCs with low constitutive expression of CYP27B1, (2) in hMSCs treated with ketoconazole, and (3) in hMSCs in which CYP27B1 expression was silenced. Osteoblast differentiation and skeletal homeostasis may be regulated by autocrine/paracrine actions of 25(OH)D<sub>3</sub> in hMSCs. © 2011 American Society for Bone and Mineral Research.

**KEY WORDS:** BONE MARROW STROMAL CELLS; VITAMIN D; PROLIFERATION; OSTEOBLAST DIFFERENTIATION; APOPTOSIS

## Introduction

Vitamin D is an important regulator of mineral and bone metabolism, and it is now appreciated that its metabolites and analogues have many other actions. Calcitriol, or 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], is the most active metabolite, with high affinity for the nuclear vitamin D receptor (VDR).<sup>(1)</sup> It is produced in the kidney by the 1 $\alpha$ -hydroxylation of the precursor 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] by the cytochrome P450 enzyme CYP27B1/1 $\alpha$ -hydroxylase.<sup>(2)</sup> Hydroxylation of vitamin D metabolites at the carbon 24 position by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) is the first step in their inactivation and excretion. Basal expression of CYP24A1 is usually low but is highly induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>.<sup>(1)</sup>

Calcitriol has major effects in inhibiting proliferation and promoting differentiation of many cell types, especially tumor cells such as

human breast cancer cells,<sup>(3)</sup> colon sarcoma cells,<sup>(4)</sup> prostate cancer cells, colorectal adenoma, and carcinoma cells.<sup>(5)</sup> Epidemiologic and experimental studies also indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub> has antitumor effects<sup>(6)</sup>; those effects are attributed to the inhibition of proliferation,<sup>(5,7,8)</sup> arrest of cell cycle,<sup>(3,9)</sup> increase in apoptosis,<sup>(4,10,11)</sup> and induction of differentiation.<sup>(12,13)</sup> The antiproliferative and prodifferentiation effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> also have been demonstrated for some nonmalignant cell types, such as human peripheral monocytes.<sup>(14,15)</sup> Little is known, however, about the effects of 25(OH)D<sub>3</sub> on cell proliferation and differentiation.

In addition to kidney tubule cells, other human cells, notably osteoblasts<sup>(16)</sup> and their progenitors in the bone marrow,<sup>(17)</sup> produce 1,25(OH)<sub>2</sub>D<sub>3</sub>. Bone cells participate in vitamin D metabolism and also are targets of 1,25(OH)<sub>2</sub>D<sub>3</sub> action.<sup>(17)</sup> The differentiation of human marrow stromal cells (hMSCs, aka mesenchymal stem cells)<sup>(18)</sup> and rat osteogenic ROS 17/2 cells<sup>(19)</sup>

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Address correspondence to: Julie Glowacki, PhD, Department of Orthopedic Surgery, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.

E-mail: jglowacki@rics.bwh.harvard.edu

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to osteoblasts is stimulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>. Less is known about the effects of 25(OH)D<sub>3</sub> on bone cells. In recent studies with freshly isolated hMSCs from 19 subjects, 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated osteoblast differentiation in all samples, and 25(OH)D<sub>3</sub> did so in two-thirds of them.<sup>(17)</sup> The variability in response to 25(OH)D<sub>3</sub> may be due to differences in expression of CYP27B1. The combined presence of CYP27B1 and VDR indicates possible autocrine/paracrine roles for 25(OH)D<sub>3</sub> in hMSCs. This study tests the hypothesis that the antiproliferative and prodifferentiation effects of 25(OH)D<sub>3</sub> in hMSCs depend on CYP27B1.

## Materials and Methods

### Cells and reagents

Bone marrow samples were obtained with institutional review board approval as femoral tissue discarded during primary hip arthroplasty for osteoarthritis. A series of samples from 22 subjects (average age is 58 ± 15 years) was prepared and screened. Low-density marrow mononuclear cells were isolated by centrifugation on Ficoll/Histopaque 1077 (Sigma, St Louis, MO, USA).<sup>(20)</sup> This procedure removes differentiated cells and enriches for undifferentiated low-density marrow mononuclear cells that include a population of nonadherent hematopoietic cells and a fraction capable of adherence and differentiation into musculoskeletal cells. The nonadherent hematopoietic stem cells were rinsed away 24 hours after seeding, and the adherent hMSCs were expanded in monolayer culture with standard growth medium, phenol red-free α modified essential medium α-MEM), 10% fetal bovine serum-heat inactivated (FBS-HI), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA). All samples were used at passages 2 through 4. Some experiments used standard osteogenic medium (ie, phenol red-free α-MEM, 10% FBS-HI, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 nM dexamethasone, 5 mM

β-glycerophosphate, and 50 μg/mL of ascorbate-2-phosphate) or osteogenic medium (ie, phenol red-free α-MEM, 1% FBS-HI, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 nM dexamethasone, 5 mM β-glycerophosphate, and 50 μg/mL of ascorbate-2-phosphate). After transfection with siRNA, all media used were without 100 U/mL of penicillin and 100 μg/mL of streptomycin. Reagents such as 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and ketoconazole were purchased from Sigma; each was prepared as a stock solution at 10<sup>-3</sup> M in absolute ethanol and stored at -80°C. In preliminary dose-finding studies (data not shown) with Western immunoblotting, we found no responses to 1, 10, or 100 nM 25(OH)D<sub>3</sub> and responses to 1000 nM 25(OH)D<sub>3</sub>; thus most experiments used 1000 nM 25(OH)D<sub>3</sub>. In all 3-day experiments, vitamin D metabolites were added daily to control for inactivation by 24-hydroxylation.

### RNA isolation and RT-PCR

Total RNA was isolated from human MSCs with TRIZOL reagent (Invitrogen). For reverse-transcriptase polymerase chain reaction (RT-PCR), 2 μg of total RNA was reverse-transcribed into cDNA with SuperScript II (Invitrogen) following the manufacturer's instructions. Concentrations of cDNA and amplification conditions were optimized for each gene product to reflect the exponential phase of amplification. One-twentieth of the cDNA was used in each 50-μL PCR reaction (30 to 40 cycles of 94°C for 1 minute, 55 to 60°C for 1 minute, and 72°C for 2 minutes), as described previously.<sup>(20)</sup> Gene-specific primer pairs (Table 1) for CYP27B1,<sup>(17)</sup> CYP24A1,<sup>(17)</sup> VDR,<sup>(17)</sup> Cbfa1/Runx2 (Runx2),<sup>(21)</sup> AlkP,<sup>(21)</sup> bone sialoprotein (BSP),<sup>(21)</sup> Bax,<sup>(21)</sup> and Bcl-2<sup>(22)</sup> were used for amplification. PCR products were separated by agarose gel electrophoresis and were quantified by densitometry of captured gel images with a Kodak Gel Logic 200 Imaging System and Kodak Molecular Imaging Software following the manufacturer's instructions (Kodak Molecular Imaging Systems,

**Table 1.** Primer Sets Used for RT-PCR

Accession number	Primer name	Sequence (5'→3')	Product size (bp)
NM_000785.3	CYP27B1	F = GCTACACGAGCTGCAGGTGCAGGGC R = AGCGGGGCCAGGAGACTGCGGAGCC	252
NM_001128915.1	CYP24A1	F = GCAGCCTAGTGAGATT R = ATTCACCCAGAAGTGTG	335
NM_001017535.1	VDR	F = AGCCTCAATGAGGAG CACTCCAAG R = ACGGGTGAGGAGGGCTGCTGAGTA	208
NM_004324.3	Bax	F = GAGGATGATTGCCCGCTGGAC R = CGGTGGTGGGGTGAGGAGG	279
NM_000633.2	Bcl-2	F = CTTTCATGTTGTGGCCGATCA R = CCCAGGGCAAAGAAATGCAAGTGA	137
NM_001015051.3	Runx2	F = GTTTGTTCTCTGACCGCTC R = CCAGTCTGAGGCACCTGAAA	318
NM_000478.4	AlkP	F = GCGAACGTATTCTCCAGACCCAG R = TTCCAAACAGGAGAGTCGCTCA	369
NM_004967	BSP	F = TCAGCATTTTGGGAATGGCC R = GAGGTTGTTGTCTTCGAGGT	657
NM_002046.3	GAPDH	F = TGATGACATCAAGAAGGTGGTGAAG R = TCCTTGAGAGCCATGTGGGCCAT	240

Rochester, NY, USA). Data were expressed by normalizing the densitometric units to *GAPDH* (internal control).

### In vitro biosynthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub> by hMSCs

For comparing synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub>, hMSCs (three replicate wells) were cultivated in 12-well plates until confluence, and then the medium was changed to serum-free  $\alpha$ -MEM supplemented with 1% insulin-transferrin-selenium plus linoleic-bovine serum albumin (ITS)<sup>+</sup>, 10  $\mu$ M 1,2-dianilinoethane (*N,N'*-diphenylethylenediamine; Sigma) and treated with or without 1000 nM 25(OH)D<sub>3</sub> for 24 hours.<sup>(17)</sup> This concentration of substrate 25(OH)D<sub>3</sub> is customary for in vitro biosynthesis studies.<sup>(17,23)</sup> 1,2-Dianilinoethane was added to the cultures as an antioxidant. Supernatants were harvested and stored at  $-20^{\circ}\text{C}$  prior to analysis for 1,25(OH)<sub>2</sub>D<sub>3</sub> content. The 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in the media were determined quantitatively with a 1,25(OH)<sub>2</sub>D<sub>3</sub> EIA kit (Immunodiagnostic Systems, Ltd., Fountain Hills, AZ, USA) according to the manufacturer's instructions. The hMSCs were lysed with a buffer containing 150 mM NaCl, 3 mM NaHCO<sub>3</sub>, 0.1% Triton X-100, and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined with the BCA System (Thermo Fisher Scientific, Rockford, IL, USA). The CYP27B1 activity was expressed as biosynthesized 1,25(OH)<sub>2</sub>D<sub>3</sub> in medium per milligram of protein per hour (femtomoles per milligram of protein per hour).

### Proliferation

Human MSCs (hMSCs<sup>hi-1 $\alpha$</sup>  and hMSCs<sup>lo-1 $\alpha$</sup> ) at passage 2 were seeded at 3000/cm<sup>2</sup> in 12-well plates. Cells were cultured in replicate (12 replicate wells) in standard growth medium (10% FBS-HI) in the absence or presence of 1, 10, or 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or 25(OH)D<sub>3</sub> for 3 days. Cells were suspended with 0.5 mL of 0.05% trypsin-ethylenediamine tetraacetic acid (Invitrogen), and cell number was determined by hemacytometer.

### Western immunoblot

Human MSCs were cultured in 100-mm dishes in standard growth medium (10% FBS-HI). At 50% confluence, the cells were treated with 1, 10, 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1000 nM 25(OH)D<sub>3</sub> for 3 days. Whole-cell lysates were prepared with lysis buffer (150 mM NaCl, 3 mM NaHCO<sub>3</sub>, 0.1% Triton X-100, and a mixture of protease inhibitors; Roche Diagnostics, Mannheim, Germany) and were homogenized with a pestle (Kontes, Vineland, NJ, USA) and centrifuged at 16,000g (Eppendorf centrifuge; Eppendorf, Hamburg, Germany). Protein concentration was determined (BCA system; Thermo Fisher Scientific). Western immunoblotting was performed as described previously.<sup>(20)</sup> In brief, proteins were resolved on 4% to 12% SDS-PAGE (NuPAGE Bis-Tris gel; Invitrogen) and transferred onto polyvinylidene fluoride membranes (PVDF; Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat milk in PBS buffer containing 0.1% Tween-20 (PBST) for 2 to 3 hours at room temperature and incubated at 4 $^{\circ}\text{C}$  overnight with primary antibodies proliferating cell nuclear antigen (PCNA) (1:3000; Abcam, Cambridge, UK), CYP27B1 (H-90, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA),  $\beta$ -actin (1:8000, Santa Cruz Biotechnology, Inc.), and Bax, Bcl-2, p21<sup>Waf1/Cip1</sup>, and cyclin D1 (each at 1:1000; Cell Signaling Technology, Beverly, MA, USA).

After removal of the unbound primary antibodies by three 5-minute washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 hour at room temperature and washed three times for 5 minutes with PBST. The antibody-associated protein bands were revealed with the ECL-plus Western blotting system (Amersham Biosciences).

### Alkaline phosphatase (AlkP) enzymatic activity assay

For AlkP enzymatic activity assay, the concentration of serum in standard osteogenic medium (10% FBS-HI) was reduced to 1% FBS-HI to minimize possible subsequent differences in proliferation that could confound interpretation of the effects of vitamin D metabolites on osteoblastogenesis. The medium was changed every 2 days. AlkP enzyme activity was measured spectrophotometrically, as described previously.<sup>(21)</sup> Protein concentration was determined with the BCA system (Thermo Fisher Scientific, Inc.). The AlkP enzyme activity was expressed as micromoles per minute per gram of protein, and some was calculated as the ratio of treated relative to control.

### RNA interference with CYP27B1 siRNA

Transient transfection of siRNA into hMSCs<sup>hi-1 $\alpha$</sup>  was performed by electroporation with the Human MSC Nucleofactor Kit (Lonza/Amaxa Biosystems, Walkersville, MD, USA) with either CYP27B1 siRNA, nonsilencing control siRNA (a nonhomologous, scrambled sequence equivalent; Santa Cruz Biotechnology, Inc.), or PBS according to the manufacturer's instructions and as described previously.<sup>(24)</sup> In brief, hMSCs<sup>hi-1 $\alpha$</sup>  were harvested by trypsinization and resuspended at 10<sup>6</sup> cells in 100  $\mu$ L of Nucleofactor Solution (Lonza/Amaxa Biosystems) with 10 or 100 pmol of CYP27B1 siRNA. Electroporation was performed in Nucleofactor II device with Program U-23 (Lonza/Amaxa Biosystems). Immediately after electroporation, the cells were transferred to 60-mm dishes or 12-well plates in phenol red-free  $\alpha$ -MEM and 10% FBS-HI. Some cells were collected at 80% confluence for RT-PCR or Western immunoblot analysis to determine the effect of CYP27B1 siRNA. Some cells that were cultured until confluent in the 12-well plates were treated with or without 1000 nM 25(OH)D<sub>3</sub> in serum-free  $\alpha$ -MEM supplemented with 1% ITS<sup>+</sup>, 10  $\mu$ M 1,2-dianilinoethane (*N,N'*-diphenylethylenediamine) for 24 hours to assess 1 $\alpha$ -hydroxylase activity. Cellular 1,25(OH)<sub>2</sub>D<sub>3</sub> production was determined by EIA as described under "In vitro biosynthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub> by hMSCs." At 24 hours after electroporation, some cells were treated with either 25(OH)D<sub>3</sub> (1000 nM) or vehicle control (ethanol) daily in standard growth medium (10% FBS-HI) for another 72 hours for RT-PCR assays. When some cells in the 12-well plates were nearly 80% confluent, the medium was changed to the osteogenic medium with 1% FBS-HI  $\pm$  10 nM 25(OH)D<sub>3</sub> for 7 days for assessment of alkP enzymatic activity as another index of osteoblast differentiation.

### Statistical analysis

Experiments were performed at least in triplicate. Group data are presented as mean  $\pm$  SEM unless otherwise indicated. Quantitative data were analyzed with nonparametric tools, either the Mann-Whitney test or Spearman correlation test. If data allowed,

parametric tools were used, either *t* test for two group or one-way ANOVA for multiple group comparisons or Pearson correlation test. A value of *p* < .05 was considered significant.

## Results

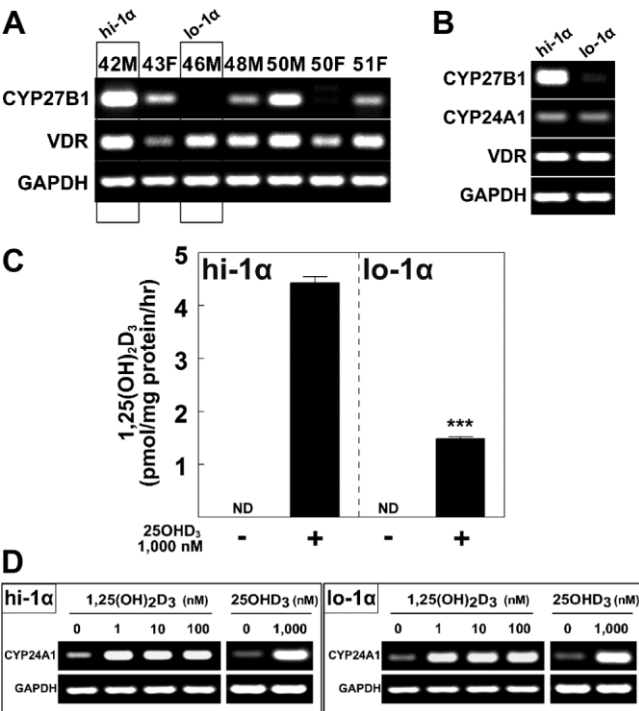
### Expression of *CYP27B1* and *CYP24A1* genes and $1\alpha$ -hydroxylase activity in hMSCs

Gene expression analysis with hMSCs from 22 subjects showed a wide range of constitutive expression of *CYP27B1* (Fig. 1A, showing 7 representative samples). Two samples of hMSCs were selected for detailed studies, having either high (hMSCs<sup>hi-1 $\alpha$</sup> , from a 42-year-old man) or low (hMSCs<sup>lo-1 $\alpha$</sup> , from a 46-year-old man) levels of *CYP27B1*, with equivalent expression of *CYP24A1* and *VDR* (Fig. 1B). Their activity for  $1\alpha$ -hydroxylation was compared by measuring production of  $1,25(\text{OH})_2\text{D}_3$ . Biosynthesis of  $1,25(\text{OH})_2\text{D}_3$  in the hMSCs<sup>hi-1 $\alpha$</sup>  was 2.98-fold greater than in the hMSCs<sup>lo-1 $\alpha$</sup>  (4433.4 versus 1487.9 fmol/mg protein per hour,

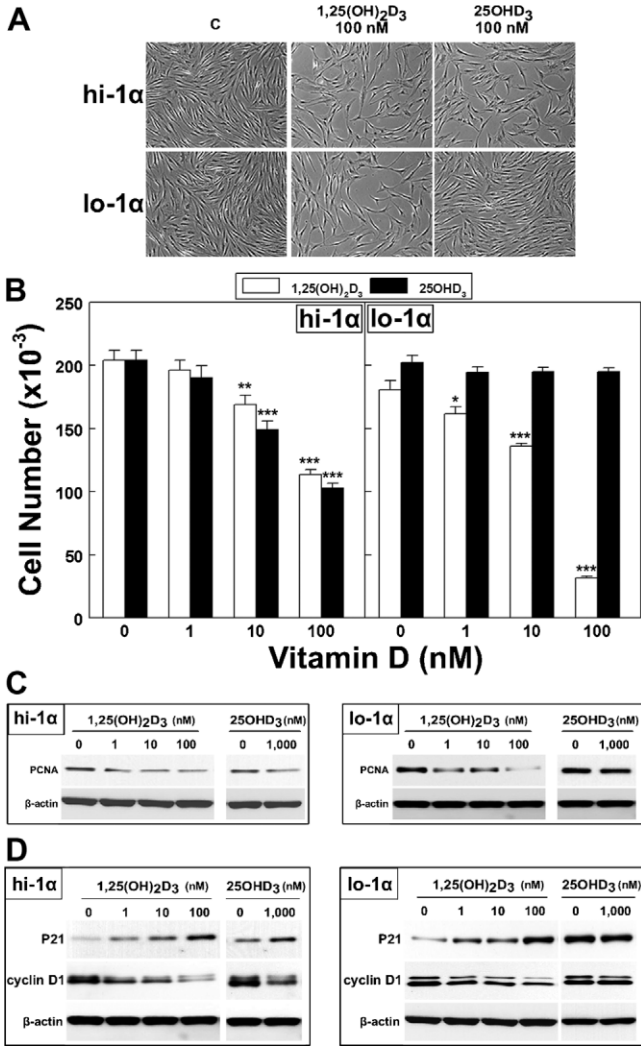
*p* < .0001; Fig. 1C). Upregulation of *CYP24A1* by  $1,25(\text{OH})_2\text{D}_3$  (1, 10, and 100 nM) or  $25(\text{OH})\text{D}_3$  (1000 nM) treatment was equivalent in both specimens of hMSCs (Fig. 1D).

### Relative antiproliferative effects of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ on hMSCs

Two samples of hMSCs were cultured for 3 days after seeding in standard growth medium (10% FBS-HI). There was less cellularity in cultures of both hMSCs<sup>hi-1 $\alpha$</sup>  and hMSCs<sup>lo-1 $\alpha$</sup>  treated with 100 nM  $1,25(\text{OH})_2\text{D}_3$  compared with vehicle control. In contrast, only the hMSCs<sup>hi-1 $\alpha$</sup>  were inhibited by  $25(\text{OH})\text{D}_3$  (Fig. 2A). There



**Fig. 1.** Expression of *CYP27B1* and *CYP24A1* genes and  $1\alpha$ -hydroxylase activity in hMSCs. (A) Gel electrophoretogram shows RT-PCR products *CYP27B1*, *VDR*, and *GAPDH* in 7 representative specimens of hMSCs. Labels for lanes indicate age and gender. (B) Gel electrophoretogram shows RT-PCR products for *CYP27B1*, *CYP24A1*, *VDR*, and *GAPDH* in selected hMSCs<sup>hi-1 $\alpha$</sup>  (from a 42-year-old man) and hMSCs<sup>lo-1 $\alpha$</sup>  (from a 46-year-old man). (C)  $1,25(\text{OH})_2\text{D}_3$  synthesis was measured in hMSCs<sup>hi-1 $\alpha$</sup>  and hMSCs<sup>lo-1 $\alpha$</sup> . Cultures were treated with or without 1000 nM  $25(\text{OH})\text{D}_3$  in serum-free  $\alpha$ -MEM supplemented with 1% ITS<sup>+</sup>, 10  $\mu\text{M}$  1,2-dianilinoethane (*N,N'*-diphenylethylene diamine) for 24 hours. Cellular  $1,25(\text{OH})_2\text{D}_3$  production (three replicate wells) was determined by EIA. There was no detectable (ND)  $1,25(\text{OH})_2\text{D}_3$  in cultures without  $25(\text{OH})\text{D}_3$  exogenous substrate. \*\*\**p* < .001. (D) Gel electrophoretogram shows RT-PCR products for *CYP24A1* and *GAPDH* in hMSC<sup>hi-1 $\alpha$</sup>  and hMSC<sup>lo-1 $\alpha$</sup>  cultures after 3 days in standard growth medium with 10% FBS-HI in the absence or presence of  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$ .



**Fig. 2.** Relative effects of  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  on proliferation of hMSCs. (A) Photomicrographs show hMSC<sup>hi-1 $\alpha$</sup>  and hMSC<sup>lo-1 $\alpha$</sup>  cultures after 3 days in the absence or presence of 100 nM  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  (×200 magnification). (B) Cell number was determined in hMSC<sup>hi-1 $\alpha$</sup>  and hMSC<sup>lo-1 $\alpha$</sup>  cultures after 3 days in the absence or presence of 1, 10, 100 nM  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$ . Results are expressed as mean  $\pm$  SEM (12 replicate wells). \**p* < .05; \*\**p* < .01; \*\*\**p* < .001. (C) Western immunoblots show proliferating cell nuclear antigen and  $\beta$ -actin levels in hMSC<sup>hi-1 $\alpha$</sup>  and hMSC<sup>lo-1 $\alpha$</sup>  cultures after 3 days in the absence or presence of  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$ . (D) Western immunoblots show p21, cyclin D1, and  $\beta$ -actin in hMSC<sup>hi-1 $\alpha$</sup>  and hMSC<sup>lo-1 $\alpha$</sup>  cultures after 3 days in the absence or presence of  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$ .

was a dose-dependent inhibition of proliferation with 1,25(OH)<sub>2</sub>D<sub>3</sub> for both cell preparations (Fig. 2B). Both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited proliferation of hMSCs<sup>hi-1α</sup>; there was a significant inhibition of proliferation of hMSCs<sup>hi-1α</sup> at 100 nM of 25(OH)D<sub>3</sub> (56% of control cell number,  $p < .001$ ) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (50%;  $p < .001$ ). In contrast, hMSCs<sup>lo-1α</sup> were resistant to 25(OH)D<sub>3</sub> (96%) yet were inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> (17%,  $p < .001$ ; Fig. 2B). Consistent with the effects on cell numbers, PCNA was downregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in hMSCs<sup>hi-1α</sup> and in hMSCs<sup>lo-1α</sup> (Fig. 2C). With 25(OH)D<sub>3</sub> treatment, PCNA in hMSCs<sup>hi-1α</sup> was 64.9% of control, but for hMSCs<sup>lo-1α</sup>, PCNA was equivalent to control. With hMSCs<sup>hi-1α</sup>, both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> downregulated cyclin D1 and upregulated the negative regulator p21<sup>Waf1/Cip1</sup> (Fig. 2D). In hMSCs<sup>lo-1α</sup>, the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell cycle regulators were similar to those for hMSCs<sup>hi-1α</sup>, but there were no effects by 25(OH)D<sub>3</sub>.

### Relative effects of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> on Bax/Bcl-2 ratios in hMSCs

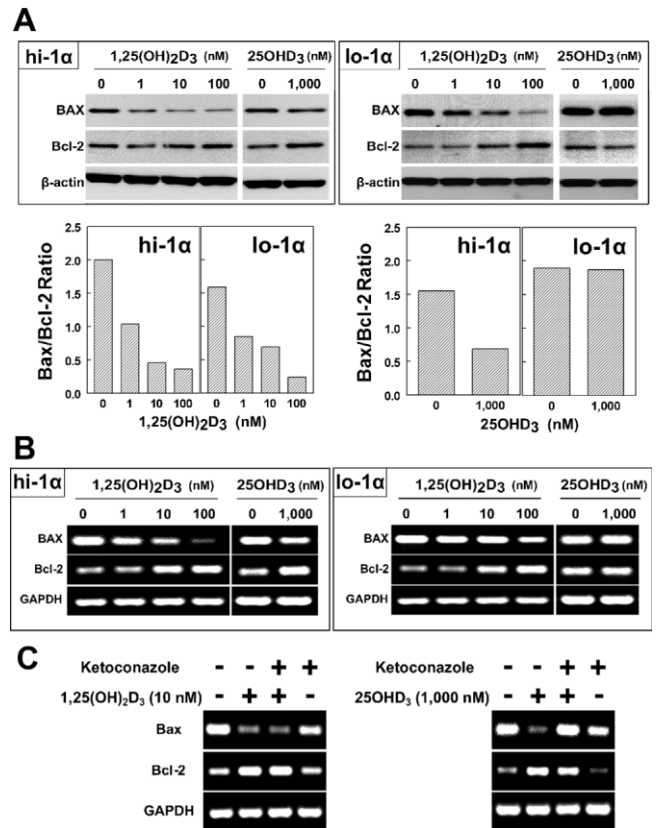
Mechanisms involved in the relative effects of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> were studied by analysis of expression of apoptosis-associated proteins. First, effects of metabolites were compared in hMSCs<sup>hi-1α</sup> and hMSCs<sup>lo-1α</sup>. In hMSCs<sup>hi-1α</sup>, both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> induced a downregulation of Bax and an upregulation of the Bcl-2 protein (Fig. 3A); these effects resulted in lower Bax/Bcl-2 ratios with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (20% compared with vehicle control) and with 1000 nM 25(OH)D<sub>3</sub> (43%). In hMSCs<sup>lo-1α</sup>, the Bax/Bcl-2 ratio was lower with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> (19%), but there was essentially no effect by 25(OH)D<sub>3</sub> (95%). The effects on mRNA levels of *Bax* and *Bcl-2* (Fig. 3B) corresponded with the changes observed for protein levels (Fig. 3A).

As a second approach, the cytochrome P450 inhibitor ketoconazole was used to determine the importance of hydroxylation on 25(OH)D<sub>3</sub> effects on proliferation. Ketoconazole (10 μM) diminished the effects of 25(OH)D<sub>3</sub> (1000 nM) and not the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM) on Bax and Bcl-2 in hMSCs<sup>hi-1α</sup> (Fig. 3C). In the presence of 25(OH)D<sub>3</sub>, the Bax/Bcl-2 ratio was 27% of that with vehicle control, and the decrease by 25(OH)D<sub>3</sub> was blocked by ketoconazole (90%). In the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, the Bax/Bcl-2 ratio was 36% of that with vehicle control, similar to that with 1,25(OH)<sub>2</sub>D<sub>3</sub> and ketoconazole (37%).

### Relative effects of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblast differentiation in hMSCs

Regulation of osteoblast differentiation was quantified first by AlkP enzymatic activity assays in osteogenic medium with 1% FBS-HI (Fig. 4A). In hMSCs<sup>hi-1α</sup>, there was similar stimulation of AlkP activity by 25(OH)D<sub>3</sub> (2.16-fold,  $p = .0003$ ) and by 1,25(OH)<sub>2</sub>D<sub>3</sub> (1.77-fold,  $p < .0001$ ). In contrast, with hMSCs<sup>lo-1α</sup>, 25(OH)D<sub>3</sub> had no effect (0.96-fold,  $p = .577$ ), and 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated AlkP activity (1.86-fold,  $p < .0001$ ).

Osteoblast differentiation was also monitored by osteoblast signature genes (ie, *Runx2*, *AlkP*, and *BSP*) after transfer to standard osteogenic medium (10% FBS-HI) or after addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> to standard growth medium (10% FBS-HI). As expected, there was time-dependent upregulation of *Runx2*, *AlkP*, and *BSP* in hMSCs<sup>hi-1α</sup> in standard osteogenic medium

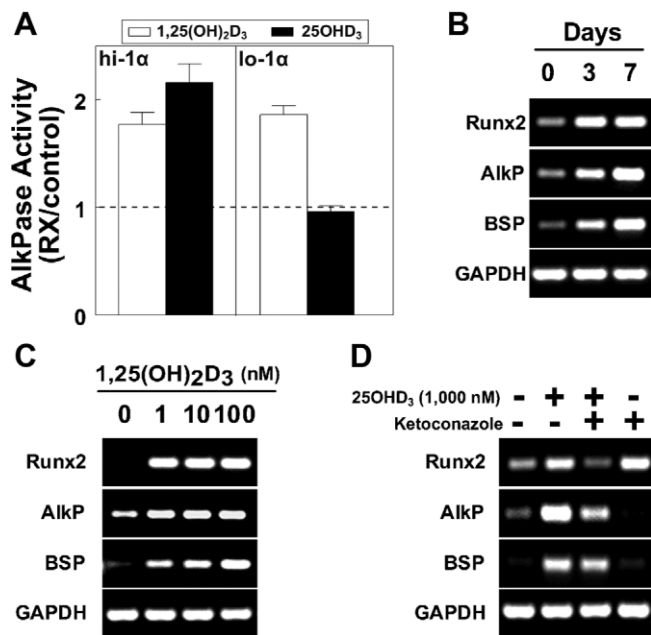


**Fig. 3.** Relative effects of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> on Bax/Bcl-2 ratios in hMSCs. (A) Western immunoblots show Bax, Bcl-2, and β-actin in hMSCs<sup>hi-1α</sup> and hMSCs<sup>lo-1α</sup> cultures after 3 days in the absence or presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> or 25(OH)D<sub>3</sub>. The bar graphs represent the Bax/Bcl-2 ratios after each densitometric value was normalized to β-actin. (B) Gel electrophoretograms show RT-PCR products for *Bax*, *Bcl-2*, and *GAPDH* in hMSCs<sup>hi-1α</sup> and hMSCs<sup>lo-1α</sup> cultures after 3 days in the absence or presence of 1, 10, or 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1000 nM 25(OH)D<sub>3</sub>. (C) Gel electrophoretogram shows RT-PCR products for *Bax*, *Bcl-2*, and *GAPDH* in hMSCs<sup>hi-1α</sup> after 3 days in the absence or presence of 10 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1000 nM 25(OH)D<sub>3</sub> ± 10 μM ketoconazole.

(Fig. 4B). Addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> to standard growth medium also upregulated osteoblast genes in hMSCs<sup>hi-1α</sup> (Fig. 4C). Addition of 25(OH)D<sub>3</sub> to standard growth medium also upregulated osteoblast genes in hMSCs<sup>hi-1α</sup>, but its effect was diminished by ketoconazole (Fig. 4D).

### Effect of CYP27B1 siRNA on the stimulation of osteoblast differentiation by 25(OH)D<sub>3</sub>

As another approach to assess the mechanism by which 25(OH)D<sub>3</sub> can stimulate osteoblast differentiation, hMSCs<sup>hi-1α</sup> were engineered to have reduced constitutive expression of CYP27B1. There were no noticeable differences in cell density or appearance of control cells (electroporation with PBS), cells treated with nonsilencing control siRNA, and cells with 10 or 100 pmol CYP27B1 siRNA (Fig. 5A). Transient transfection of CYP27B1 siRNA into hMSCs<sup>hi-1α</sup> resulted in reductions of CYP27B1 mRNA (2% of control; Fig. 5B) and CYP27B1 protein (11% of control; Fig. 5C). No effect was shown with a nonsilencing, scrambled siRNA sequence (lane NC in Fig. 5B, C). The amount of 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesized by the cells transfected with CYP27B1

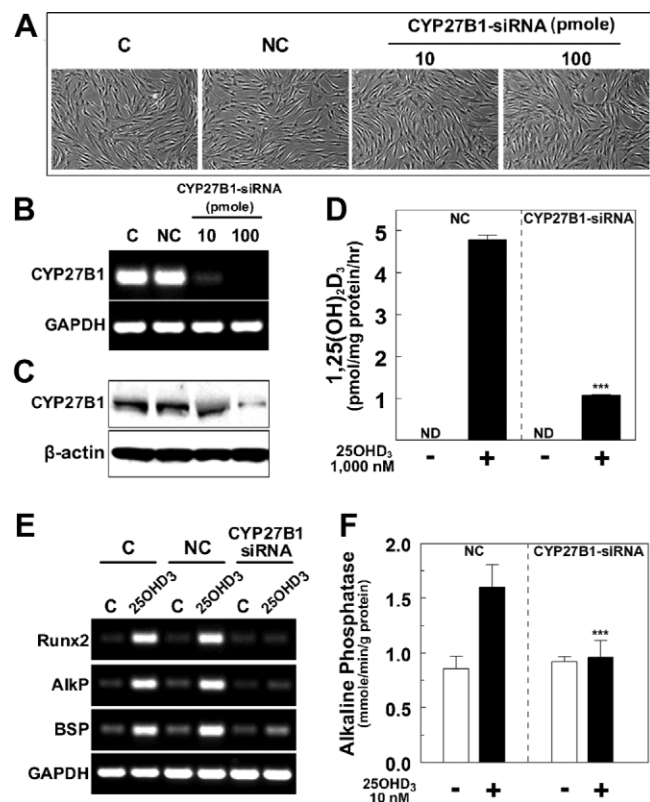


siRNA was 22% of that for cells transfected with nonsilencing siRNA (1075 versus 4786 fmol/mg protein per hour,  $p < .0001$ ; Fig. 5D). Treatment with 25(OH)D<sub>3</sub> upregulated *Runx2*, *AlkP*, and *BSP* in both control preparations of hMSCs<sup>hi-1α</sup>. With cells transfected with *CYP27B1* siRNA, however, 25(OH)D<sub>3</sub> had no effect on osteoblast genes (Fig. 5E). As a functional marker of osteoblast differentiation, we measured AlkP enzymatic activity after 7 days in osteogenic medium (1% FBS-HI). Whereas 25(OH)D<sub>3</sub> stimulated AlkP activity of control cells (1.87-fold,  $p < .0001$ ), there was no effect in cells transfected with *CYP27B1* siRNA (1.04-fold,  $p = .093$ ; Fig. 5F).

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## Discussion

This study used three approaches to examine the role of *CYP27B1* on the effects of 25(OH)D<sub>3</sub> in hMSCs. First, we compared cells with high and low constitutive expression of *CYP27B1*. Finding a wide range of expression in hMSCs from 22 subjects is consistent with our previous studies.<sup>(17)</sup> The level of



*CYP27B1* expression was found to be related to the vitamin D status<sup>(17)</sup> and, more recently, to age<sup>(25)</sup> of the subjects. There is growing evidence that hMSCs<sup>(17)</sup> and human bone cells<sup>(26)</sup> are both sources and targets of 1,25(OH)<sub>2</sub>D<sub>3</sub>, and thus vitamin D may have multiple autocrine/paracrine actions in bones. It was important to control for 24-hydroxylation in these studies because differences in inactivation of added vitamin D metabolites could confound interpretation. The activities of *CYP27B1* and *CYP24A1* are important for the maintenance of appropriate levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>. Therefore, two

hMSCs were selected and studied in detail on the basis of having extremes in expression of CYP27B1 and equivalent expression of CYP24A1 and VDR. Further, the expression of CYP24A1 was found to be regulated with equivalence in both specimens of hMSCs; this observation reduces concerns of confounding effects of 24-hydroxylation in these studies. In addition, fresh metabolites were added daily.

There were substantial differences in the synthesis of  $1,25(\text{OH})_2\text{D}_3$  by hMSCs<sup>hi-1 $\alpha$</sup>  and hMSCs<sup>lo-1 $\alpha$</sup> . The difference also held for hMSCs with and without CYP27B1 gene silencing. Although these experiments cannot be used to estimate what would be the steady-state concentration of  $1,25(\text{OH})_2\text{D}_3$  in the bone marrow in different subjects whose cells have high or low expression of CYP27B1, they provide evidence for a potential autocrine/paracrine role for  $25(\text{OH})\text{D}_3$  metabolism in osteoblast differentiation. Similar ideas have been proposed for  $25(\text{OH})\text{D}_3$  metabolism in regulating bone matrix formation by differentiated human osteoblasts.<sup>(23)</sup>

There was dose-dependent inhibition of proliferation by  $25(\text{OH})\text{D}_3$  with hMSCs that had a high level of expression of CYP27B1;  $25(\text{OH})\text{D}_3$  reduced their proliferation and down-regulated PCNA. There is some information about antiproliferative actions of  $25(\text{OH})\text{D}_3$  in other human cell types. In human primary prostate epithelial cells that expressed CYP27B1, low concentrations of  $25(\text{OH})\text{D}_3$  suppressed cell growth.<sup>(27)</sup> In prostatic cancer cells lacking CYP27B1,  $25(\text{OH})\text{D}_3$  failed to demonstrate antiproliferative action.<sup>(28)</sup> There are several mechanisms mediating the antiproliferative effects of  $1,25(\text{OH})_2\text{D}_3$ . In U937 myelomonocytic cells,  $1,25(\text{OH})_2\text{D}_3$  induces an arrest in the G<sub>1</sub> phase of the cell cycle that depends on upregulation of the cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup>.<sup>(29)</sup> More recently, p21<sup>Waf1/Cip1</sup> was shown to be a primary antiproliferative mediator for the VDR in the presence of its ligand,  $1,25(\text{OH})_2\text{D}_3$ .<sup>(30)</sup> Cyclin D1 is increased in dividing cells during the G<sub>1</sub> phase and is necessary for the transition from G<sub>1</sub> to S phase.<sup>(31)</sup> Vitamin D decreases cyclin D1 abundance and/or activity by different mechanisms in different cell types. For example, in human epidermoid A431 cells,  $1,25(\text{OH})_2\text{D}_3$  inhibited transforming growth factor  $\alpha$  (TGF- $\alpha$ )/endothelial growth factor receptor (EGFR) transactivation of cyclin D1.<sup>(32)</sup> We found that  $25(\text{OH})\text{D}_3$  downregulated cyclin D1 and upregulated the negative regulator p21<sup>Waf1/Cip1</sup> in hMSCs<sup>hi-1 $\alpha$</sup> . In contrast,  $25(\text{OH})\text{D}_3$  had no such effects in hMSCs<sup>lo-1 $\alpha$</sup> . The upregulation of p21<sup>Waf1/Cip1</sup> and decreased expression of cyclin D1 in hMSCs<sup>hi-1 $\alpha$</sup>  provide mechanisms for the antiproliferative effect of  $25(\text{OH})\text{D}_3$ .

$1,25(\text{OH})_2\text{D}_3$  also affects the levels of proapoptotic (ie, Bax and Bak) and antiapoptotic (ie, Bcl-2 and Bcl-XL) proteins, resulting in apoptosis in several tumor models, including human carcinomas of the breast, colon, and prostate.<sup>(4,10,33)</sup> This study with hMSCs indicates that the antiproliferation effects of  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  are not explained by increases in Bax or decreases in Bcl-2. In fact, the ratio of Bax/Bcl-2 decreases at both the mRNA and protein levels. Two lines of evidence indicate that those effects of  $25(\text{OH})\text{D}_3$  on Bax and Bcl-2 depend on CYP27B1. First, they were not detected in hMSCs<sup>lo-1 $\alpha$</sup> . Second, they were blocked in hMSCs<sup>hi-1 $\alpha$</sup>  by the pan-cytochrome P450 inhibitor ketoconazole, not like the effects of  $1,25(\text{OH})_2\text{D}_3$ , which were not affected by ketoconazole. The antiapoptotic effects of  $1,25(\text{OH})_2\text{D}_3$  or

$25(\text{OH})\text{D}_3$  in hMSCs<sup>hi-1 $\alpha$</sup>  are different from the proapoptotic effects in some cancer cells<sup>(4,10,33)</sup> and are similar to the effects in other cell types. In ovarian cancer cells,  $1,25(\text{OH})_2\text{D}_3$  inhibits apoptosis that is mediated by death receptors.<sup>(34)</sup> In rat osteoblast-like osteosarcoma UMR 106 cells,  $1,25(\text{OH})_2\text{D}_3$  elicited antiapoptotic effects by decreasing the Bax/Bcl-2 ratio.<sup>(11)</sup> There are other antiapoptotic signals, as was reported for nongenotropic mechanisms in osteoblasts and osteocytes.<sup>(35)</sup> In sum, the data indicate that the antiproliferative effects of  $25(\text{OH})\text{D}_3$  in hMSCs<sup>hi-1 $\alpha$</sup>  and of  $1,25(\text{OH})_2\text{D}_3$  in both samples of hMSCs are explained by cell cycle arrest and not by increased apoptosis.

Calcitriol induces differentiation of many types of benign and malignant cells.<sup>(12,13,18,36–38)</sup> The differentiation of various cells, including human myelomonocytic cells,<sup>(9,29)</sup> induced by  $1,25(\text{OH})_2\text{D}_3$  depends on the induction of p21<sup>Waf1/Cip1</sup>. Further,  $1,25(\text{OH})_2\text{D}_3$  is a key regulator of the reciprocal relationship between proliferation and differentiation during the osteoblast development sequence.<sup>(39)</sup> Vitamin D or its analogues promote osteoblastic differentiation, as shown for osteosarcoma cell lines MG-63,<sup>(40)</sup> HOS,<sup>(23)</sup> SAOS,<sup>(41)</sup> and TE85.<sup>(41)</sup> Osteoblast differentiation of human MSCs is stimulated by  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  in hMSCs<sup>hi-1 $\alpha$</sup> , as shown here and elsewhere.<sup>(17,18,37,38,42)</sup> As expected,  $25(\text{OH})\text{D}_3$  failed to stimulate osteoblast differentiation in hMSCs<sup>lo-1 $\alpha$</sup> . Upregulation of osteoblast genes by  $25(\text{OH})\text{D}_3$  in hMSCs<sup>hi-1 $\alpha$</sup>  was diminished by ketoconazole. Thus experiments with ketoconazole indicate that both the antiproliferative and prodifferentiation effects of  $25(\text{OH})\text{D}_3$  depend on CYP27B1.

Ketoconazole is a strong but differential inhibitor of both CYP24A1 and CYP27B1<sup>(43)</sup> and may be cytotoxic for some cells.<sup>(23)</sup> Those confounders may complicate interpretation of results obtained with this agent. We therefore used the highly specific technique of RNA interference to inhibit CYP27B1 expression in hMSCs<sup>hi-1 $\alpha$</sup> . The level of synthesized  $1,25(\text{OH})_2\text{D}_3$  in the cells transfected with CYP27B1 siRNA was reduced to 22% of that for cells transfected with nonsilencing siRNA. Osteoblast differentiation of hMSCs<sup>hi-1 $\alpha$</sup>  by  $25(\text{OH})\text{D}_3$  was prevented upon transfection with CYP27B1 siRNA, as indicated by osteoblast signature gene expression and by AlkP enzymatic activity. These findings are consistent with those from a study with HOS human osteosarcoma cells in which silencing of CYP27B1 resulted in a suppression of  $25(\text{OH})\text{D}_3$ 's effects on those cells.<sup>(23,44)</sup>

In conclusion,  $25(\text{OH})\text{D}_3$  has multiple effects in normal hMSCs; it inhibits proliferation and promotes osteoblast differentiation by mechanisms similar to those for  $1,25(\text{OH})_2\text{D}_3$ . Our data indicate that antiproliferative and prodifferentiation effects of  $25(\text{OH})\text{D}_3$  in hMSCs require  $1\alpha$ -hydroxylase. There are suggestions that other effects of  $25(\text{OH})\text{D}_3$  in other cell types, such as induction of 24-hydroxylase in prostatic cells, may not require  $1\alpha$ -hydroxylase.<sup>(45)</sup> Three lines of evidence indicate that CYP27B1 is required for the effects of  $25(\text{OH})\text{D}_3$  on hMSCs. Those effects were not seen (1) in hMSCs with low constitutive expression of CYP27B1, (2) in hMSCs treated with ketoconazole, or (3) in hMSCs in which CYP27B1 expression was silenced. These findings suggest that local osteoblast differentiation in vivo may be promoted by  $25(\text{OH})\text{D}_3$  if the progenitor/precursor cells in marrow express CYP27B1/ $1\alpha$ -hydroxylase. We found that many of hMSCs' in vitro behaviors and baseline characteristics depend on clinical features of the subjects from whom the cells were



isolated. The level of CYP27B1 expression in those cells depends on vitamin D status and can be regulated by a number of factors, including vitamin D.<sup>(17)</sup> The combined presence of CYP27B1 and the VDR in hMSCs indicates possible autocrine/paracrine roles for 25(OH)D<sub>3</sub> to regulate osteoblast differentiation and skeletal homeostasis.

## Disclosures

All the authors state that they have no conflicts of interest.

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